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Dielectric relaxation study of Lysozyme in Water/Ethylene-Glycol solution using time domain reflectometry (TDR) technique.

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ABSTRACT

This work deals with a dielectric study at microwave frequencies of the influence at room temperature of organic molecules, known as, ethylene-glycol on conformational and dynamic properties of lysozyme from chicken egg-white lysozyme is composed of two structural domains, separated by the active site cleft. Measurements were carried out at the fixed temperature of 25^{0}C varying the concentration of the co-solvent up to 90% v/v. From the analysis of the dielectric relaxation of the protein solution, the effective hydrodynamic radius and the electric dipole moment of the protein were calculated as a function of the co-solvent concentration. On the contrary ethylene-glycol strongly affects the dielectric response of lysozyme denoting a specific effect on its conformation and dynamics. The data are coherently interpreted hypothesizing that glycol molecule wedges between and separates the two domains of lysozyme making them rotationally independent. We have investigated the dielectric properties, as a function of frequency and temperature, of the hydrated globular protein lysozyme. A dielectric measurement was performed on lysozyme using dielectric time domain spectroscopy over a wide range of frequency 10 MHz to 30 GHz. The static dielectric constant, relaxation time and conductivity have been determined using nonlinear least squares fit method. The hydration numbers were also determined from the static dielectric constant.

Keywords:- dielectric constant, relaxation time, conductivity, hydration number, time domain reflectometry.

1. INTRODUCTION

Enzymes catalyse many biological reactions. The rates of chemical reaction in the presence of enzymes are, in some cases, accelerated more than 10 orders of magnitude relative to the corresponding rates in solution [1]. The origins of these catalytic effects have been studied extensively for many years. One hypothesis is that enzymes reduce the activation barrier by removing water molecules and providing a gas phase like environment inside the enzymes [2, 3]. It has been argued that this hypothesis contrasts with the proposed role of the enzyme active site which provides favourable electrostatic interactions, such as hydrogen bonding, between the protein and the transition state. It has been proposed that enzymes may act by providing a specific polar environment that is different from the gas phase [4,5]. However, the electrostatic interactions, in a low dielectric medium are stronger than those in a high dielectric medium. For example, the strength of the specific hydrogen bond in a polar solvent is quite weak. Therefore, it can be proposed that enzymes may provide not only specific electrostatic interactions such as hydrogen bonding, dipole-dipole and ion-dipole interactions, but also the low dielectric environment in which they are maximized. The relative permittivity in proteins has long been thought to be low, but the fact that the pKa values of amino acid side chains in proteins are similar to the corresponding pKa values in water provides indirect evidence against this [6,7].

This work deals with a dielectric study at microwave frequencies of ethylene-glycol influence on dynamic properties of lysozyme, representing very useful models for folding studies: lysozyme from chicken egg-white [8]. The role played by water molecules adsorbed on the surface of a protein is crucial for processes ranging from enzymatic activity [9] and catalysis in organic solvents [10] to anhydrobiosis [11, 12]. A convenient experimental system to study, on one hand, the dynamics of adsorbed water molecules and, on the other, to correlate these dynamical features with biological functions is represented by a hydrated powder of a globular protein, such as lysozyme [13]. In particular, the lysozyme– water system has been studied by several experimental techniques [14-17]. The conductivity percolative transition found for the lysozyme–water system is due to proton displacements along hydrogen-bonded water molecules adsorbed on the protein surface, with ionizable groups as sources of migrating protons [17, 18]. The dynamics of these migrating protons over the

lysozyme surface has been investigated by means of conventional dielectric techniques over a limited frequency and temperature range [19, 20] and recently by means of broadband dielectric spectroscopy [21]. Both experiments provided evidences of interesting analogies with the dielectric behavior of fragile proton glasses, the electric counterparts of magnetic spin glasses. In particular, the three canonical features of a glassy system [22] such as non-Arrhenius temperature dependence of the dielectric relaxation time, non-exponential relaxation processes, along with non-ergodic behavior below a transition temperature were observed.

Dielectric spectroscopy (DS) is a noninvasive, very sensitive technique to investigate complex systems and it is particularly suitable in studying biological systems. Protein solutions exhibit at radio frequencies typical dielectric relaxations due to orientation polarization. From the dispersion curve it is possible to determine two significant parameters characterizing conformation and structure of a protein: the effective hydrodynamic radius and the electric dipole moment. Our experiments have shown that dielectric spectroscopy is really a valid tool in studying structural and conformational modifications of proteins promoted by different agents, such as pH, temperature, and solvent composition. The technique is highly sensitive and able to evidence small effects on the overall conformation of the macromolecule [23].

In this paper, we report on ample study of the lysozyme at different concentration using time domain reflectometry technique in the frequency range of 10 MHz to 30 GHz [24].

2. EXPERIMENTAL DETAILS

The ethylene glycol used in these experiments was purchased from Qualigens fine chemical and lysozyme was purchased from Himedia Laboratory Pvt. Ltd. Water mixtures of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% v/v concentration in ethylene glycol were prepared. Distilled and deionized water with electric conductivity lower than $18.3\mu s^{-1}$ was obtained from an ultra-pure water distiller (Millipore, MILLI- Q Lab).

We utilize a time domain reflectometry (TDR) technique for measuring dielectric spectra of liquids up to 30GHz with a Tektronix DSA8200 Digital Serial Analyzer Oscilloscope mainframe along with the sampling module 80E08. A flat ended coaxial line makes contact with the liquid sample, and TDR measure the complex reflection coefficients at frequencies between 10 MHz to 30 GHz. A repetitive fast rising 250mv voltage pulse of 200 KHz with 18ps incident rise time was fed through coaxial line system of impedance 50 ohm. The co-axial cable was copper metal and semi rigid model no. EZ_86/M17 (Huber Suhner Electronics Pvt. Ltd.). The inner diameter of outer conductor is 2.2mm; outer diameter of center conductor is 0.51mm and the diameter of dielectric material is 1.68mm. Sampling oscilloscope monitors changes in step pulse after reflection from the end of line. Reflected pulse without sample $R_1(t)$ and with sample $R_x(t)$ were recorded in time window of 5ns and digitized in 2000 points. The complex permittivity spectra have been obtained from reflection coefficient spectra by applying the least squares fit method. The details of the apparatus and the procedures of the TDR have been reported previously [25-34].

3. RESULT AND DISCUSSION

The complex permittivity spectra of lysozyme at various concentrations are shown in figure 1. It is seen from the figure 1 that the real part of permittivity decreases with increase in frequency. The complex permittivity spectra were fitted to Havriliak-Negami equation using least square fit method [35].where ε_0 is the static dielectric constant, ε_∞ is the permittivity at high frequency, τ is the relaxation time, σ is the dc conductivity of the solution, which is essentially due to free ions, and $\in_v = 8.85 \times 10^{-12}$ F/m is the permittivity of vacuum. The α and β are the empirical parameters for the distribution of relaxation times with values between 0 and 1, with ε_0 , ε_∞ , τ , α , β and σ as fitting parameters in equation 1. The Havriliak Negami equation includes three relaxation models as limiting forms: the Cole-Cole (β =1, $0 \leq \alpha \leq 1$), Davidson-Cole (α =0) and $0<\beta<1$ and Debye (α =0, β =1) relaxation models [36]. For the systems studied here, the values of α and β are found to be 0 and 1 respectively within experimental error. The resulting values of dielectric relaxation parameters for aqueous protein solution at different pH are listed in Table 1.

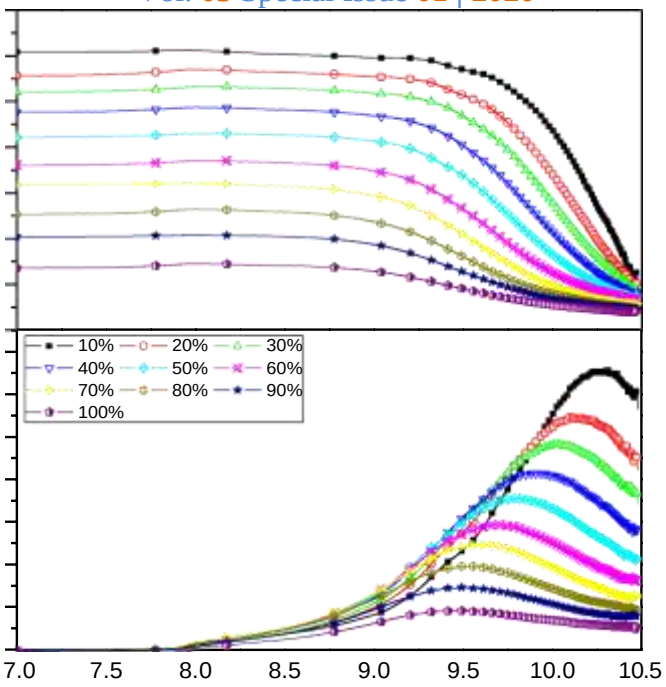

Fig. 1 Complex permittivity spectra vs Log frequency (Hz) of lysozyme in various concentrations at 25^{0}C.

Table 1. Dielectric relaxation parameters of lysozyme at 25^{0}C. The number of bracket denotes uncertainties in the last significant digits obtained the least squares fit method e. g. 59.46 (2) means 59.46 □ 0.02.

C (%)	ε_∞	ε_0	τ(ps)	σ (mho/m)	β
10	1.85(1)	59.46(2)	10.40(2)	0.07620(7)	0.982(2)
20	1.89(1)	56.53(1)	13.93(3)	0.05483(6)	0.938(1)
30	2.00(1)	53.52(2)	16.96(5)	0.04064(5)	0.915(1)
40	2.12(2)	49.36(3)	21.33(9)	0.03426(4)	0.888(1)
50	2.18(2)	44.13(5)	26.19(18)	0.02398(3)	0.865(2)
60	2.50(3)	37.87(9)	32.30(37)	0.01868(2)	0.833(4)
70	2.82(2)	33.43(8)	41.20(41)	0.01504(2)	0.828(3)
80	2.85(2)	27.79(10)	47.85(59)	0.008514(1)	0.804(4)
90	3.05(1)	23.02(9)	51.23(60)	0.005061(1)	0.773(4)
100	3.16(1)	17.26(10)	55.74(71)	0.000839(1)	0.759(5)

Table 2. Protein hydration information extracted from the dielectric measurements at 25^{0}C.

C (M)	Density (gm/c^3)	VD (nm^3)	W_{hyd}	N_{hyd}
1.0965	0.99687	291.08(8)	10.96	8890 ± 250
2.1930	0.98822	170.49(4)	5.73	4653 ± 129
3.2895	0.98956	131.22(3)	4.24	3440 ± 92
4.3860	0.98722	117.26(3)	3.66	2976 ± 76
5.4825	0.98055	113.65(3)	3.46	2813 ± 69
6.5789	0.96504	115.67(3)	3.42	2778 ± 66
7.6754	0.97521	112.60(2)	3.43	2788 ± 64
8.7719	0.96670	114.30(2)	3.46	2810 ± 64
9.8684	0.94585	114.15(2)	3.33	2709 ± 61
10.9649	0.92750	117.20(2)	3.38	2747 ± 61

We assumed that the Lysozyme in solution is spherical and has spherical hydration shells and solvent outside of hydrated Lysozyme has the same dielectric properties as that of water as shown in figure 2.

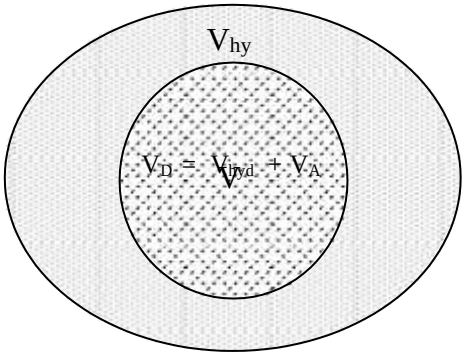

Figure 2. Illustration of the model for the dielectric excluded volume (V_D), the effective displacement volume (V_A) of the Lysozyme, and the volume of the hydrated water (V_{hyd})

Therefore, the microwave dielectric excluded volume V_D is assumed to have a dielectric constant [32] of $\varepsilon_{ex} = \varepsilon_{\omega\infty} = 5.0$. The effective permittivity of voids in bulk water may be modeled by electrostatic theory. We define the dielectric excluded volume fraction R_V as the total volume of the hydrated protein molecule (V_D) per liter of the solution. The permittivity of the solution can be described by the Maxwell- Wagner formula [33, 37].

$$\varepsilon(\omega) = \varepsilon_{(\omega)} \frac{2(1-R_V)\varepsilon_w(\omega) + (1+2R_V)\varepsilon_{ex}}{(2+R_V)\varepsilon_w(\omega) + (1-R_V)\varepsilon_{ex}^{s}} \qquad (2)$$

where R_V is the Volume fraction of protein and is as follows:

$$R = \frac{(\varepsilon_{w0} - \varepsilon_{s0})(2\varepsilon_{w0} + \varepsilon_{w})}{(3)^{V}(2\varepsilon + \varepsilon)(\varepsilon - \varepsilon)}$$

$R_V = 0$ if the dielectric constant of the voids is $\varepsilon_{w0}(\varepsilon_{s0} \equiv \varepsilon_{w0})$ and $R_V=1$ when $\varepsilon_{s0} = \varepsilon_{w\infty}$. The excluded volume of proteins is determined as follows [37].

and

$$V_D = R_V c N_0 (4)$$

where,

ε_{w0} =78.3 and $\varepsilon_{w\infty}$ = 5.0 are taken for the static and high-frequency dielectric constants of pure water at 25^0C, c is the molar protein concentration, and N_0 is the Avogadro's constant.

The assumption that the volume occupied by a water molecule is independent of the concentration is supported by the observed linearity of the density versus concentration. The difference between the densities of protein solutions (d) and pure water (do) is the difference between the mass of solute added and the mass of solvent displaced per unit volume as shown in figure 3. This suggests that tight packing of water in the hydration shell partially compensates for the displacement of solvent by the solute molecule.

$$W_{fw} = (1 - R_V)d_0 \qquad (5)$$

where, d_0 is the density of the solvent (for bulk water at 25^0C, d_0 = 0.99707 Kg/L). Therefore, the total mass of water in l L of the solution can be found as

$$W = d - 10^{-3} Mc \qquad (6)$$

where, M is the molecular weight of protein, d is the solution density and c is the protein concentration. The molecular weight of Lysozyme is 14,600 Da. The difference between the total mass of water and that of the free water is the mass of hydrated water per liter of the solution. Therefore, the number of hydrated water molecules for each Lysozyme molecules is given by

$$N_{hyd} = (W_w - W_{fw}) / (0.018c)$$

and the weight ratio of bound water mass to protein mass is

$$W_{hyd} = 18N_{hyd} / M \quad (7)$$

The hydration number is determined by the charge density at the protein surface, which progressively decreases. As a consequence the protein-water interaction is progressively weaker, with fewer water molecules bound by Coulomb forces in the Lysozyme solution. The estimated dielectric excluded volume per Lysozyme molecule, number of hydration water molecules, and derived weight ratio are listed in Table

2. The average number of hydration water molecules per protein molecule is shown in figure 4.

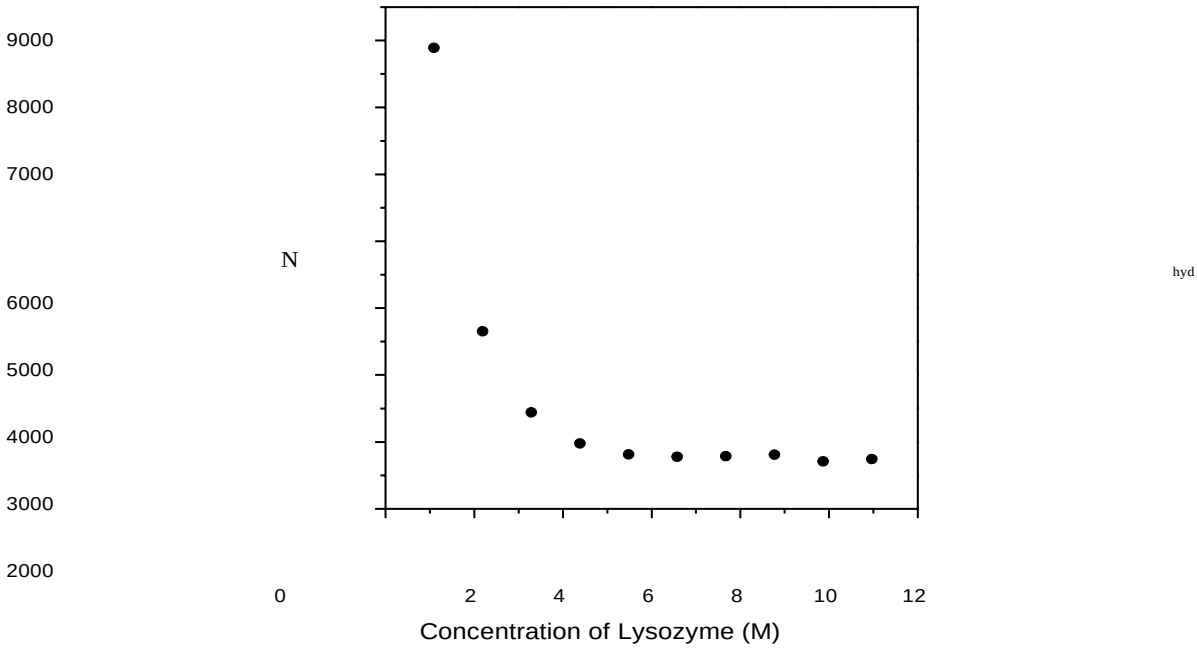

Figure 4. Hydration number with concentration (M) for Lysozyme.

4. Protein Hydration

The term hydration is commonly used to cover two different phenomena: (a) the total interaction of a solute with its aqueous solvent environment; and (ii) the perturbation of the structure and dynamics of bulk water caused by the interaction with the solute. Here, we use hydration in the latter, more restrictive sense. The water molecules that interact with a protein can, with little ambiguity, is classified as internal or external. Internal water molecules occupy cavities within the protein and are present in most globular proteins [38, 39]. They are conserved to the same extent as the amino acid sequence and must therefore be essential for function [40]. For most purposes, internal water molecule are best regarded as an integral part of the protein, even though they exchange with external water molecules, typically on time scale of 0.1-1.0 μs [41].

Protein-water interactions are of similar strength as water-water interactions and are therefore not expected to induce extensive structural perturbations. Indeed, magnetic relaxation [41] and computer simulation [42-44] studies indicate that only water molecules in direct contact with the protein surface are significantly perturbed. Moreover, the vast majority of water molecules in this hydration layer are not more perturbed that water molecules in contact with small solute [45]. Nevertheless, these water molecules are often referred to as 'bond'. This term appropriately describe the strongly exothermic adsorption of water molecules on the surface of a dry (vaporized or lyophilized) protein [46], but it is misleading when applied to a protein immersed in an aqueous solvent. Water molecules in the hydration layer of a dissolved protein are not bound in a thermodynamic or kinetic sense. It is therefore not physically meaningful to describe protein hydration in terms of equilibrium between bound and free water as is commonly done [47-49]. In the absence of co solvents, every exposed hydration site is virtually always occupied by a water molecule from the 'bond' to the 'free' state is invariably accompanied by the reverse transition of another water molecule. In other words, we are dealing with a symmetric exchange process for which the equilibrium constant is trivially equal to one. Water simply fills the available space.

5. Discussion of hydration Numbers:

The experimental quantity that is directly obtained form the analysis of the measurements is the microwave dielectric excluded volume V_D per protein molecule as shown in table 2. The important is that, for native Lysozyme, this volume is larger than the bare molecular volume in the crystal, V_{Lyso}~291 nm^3 [50]. The excess is attributed to the hydration shell, and knowing V_D, the weight ration W_{hyd} and Hydration number N_{hyd} are easily obtained.

The weight ratio obtained frequency measurements; weight ratio values obtained ranges from 11 – 03 for Lysozyme seem to be varying with concentration as it showed decreasing trend with increasing concentration of solution. The average value for N_{hyd} of Lysozyme found to be 96.

Using spheres with r_{Lyso} = 41.11 A^0 to approximate Lysozyme at 1.0965 M concentration. The average molecular size of bulk water molecules, with density d_w = 1, is 3.1 A^0.

From calculations derived it can be inferred that hydration number N_{hyd} derived from direct dielectric measurements is greater. The above estimate suggest that the hydration shell corresponds roughly to a single atomic layer of water surrounding Lysozyme molecules i.e. 'bound water' that is more tightly packed that bulk water. Increased N_{hyd} form dielectric calculations may contributed by free water. From previous studies [37, 51], it is clear that buffer ions will also bind water molecules, therefore deionized pure water was used in study with buffer having very low molarity sufficient for imparting charges on surface of Lysozyme.

6. Conclusion

In this work we compared the different concentrations of water/ethylene glycol with lysozyme at 25^0C temperature. On conformational and dynamical properties of Lysozyme, with distinctive structural properties. Data shows that glycerol does not modify significantly the conformation of lysozyme. On the contrary ethylene-glycol strongly affects the dielectric response at microwave frequencies of lysozyme, denoting a relevant structural effect.

The dielectric dispersion of Lysozyme has been studied at various concentrations using time domain reflectometry technique. The static dielectric constant, relaxation time, d.c. conductivity, hydration number has been determined. The dielectric data reveal the formation of a solvation sheath of bound water molecules around the Lysozyme. The present study helps to determine interaction of Lysozyme with its ligand at different concentrations using dielectric property and hydration number study.

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